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Construction and Characterization of Herpes Simplex Virus Type 1 Mutants with Conditional Defects in Immediate Early Gene Expression

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The herpes simplex virus type 1 (HSV-1) mutant *in*1814 contains an insertion mutation in the coding sequence for the virion transactivator protein VP16 and is thus impaired for the activation of immediate early (IE) gene expression. This virus was modified further by introducing the Moloney murine leukemia virus LTR promoter in place of the upstream sequences controlling expression of the IE regulatory protein ICP0, to yield mutant *in*1820. In almost all cell types tested, *in*1820 initiated infection less efficiently than *in*1814, behaving as if lacking both VP16 and ICP0 functions, but in BHK cells *in*1820 was less impaired than *in*1814. A rescuant of *in*1820 at the VP16 locus, *in*1825, also exhibited a host range phenotype, initiating replication as efficiently as wild-type HSV-1 in BHK cells but inefficiently in other cell types. *In*1825 was unable to complement an ICP0 null mutant in restricted cells, demonstrating that the promoter exchange prevented the expression of ICP0 protein in functionally significant amounts. The novel host range properties of *in*1820 provided a basis for the construction of additional viruses conditionally impaired for IE gene expression and assessment of their value as prototype vectors. Production of an HSV-1 mutant multiply defective in the expression of IE gene products was achieved by introduction of the temperature-sensitive mutation of HSV-1 *ts*K, which inactivates the IE transcription activator ICP4 at nonpermissive temperatures, into *in*1820 to produce *in*1820K. This mutant could be propagated effectively in BHK cells at 31° but was effectively devoid of the major regulators ICP0, ICP4, and VP16 in other cells types at 38.5°. Cultures could withstand infection with 5 PFU of *in*1820K per cell without detectable cytopathology and could be reseeded to form colonies at approximately 90% efficiency. A derivative of *in*1820K containing the *Escherichia coli* lacZ gene controlled by the human cytomegalovirus (HCMV) major IE promoter expressed low but detectable levels of β-galactosidase in almost all cells after infection of cultures at 5 PFU per cell and incubation at 38.5°. Cultures infected with 5 PFU per cell of an *in*1820K derivative expressing neomycin phosphotransferase (npt) controlled by the HCMV IE promoter were resistant to killing by the antibiotic G418 for up to 3 days, and cell survival correlated with the retention of functional levels of npt. Mutants based on *in*1820K can thus express foreign gene products in virtually all cells in a culture under conditions in which cytotoxicity is eliminated, demonstrating that progressive reduction of IE gene expression is an important step in the design of HSV-1-derived vectors.

INTRODUCTION

Infection of permissive cells with herpes simplex virus type 1 (HSV-1) results in the coordinated expression of viral gene products. The five genes usually regarded as immediate early (IE) (and also the UL39 gene) are transcribed in the absence of *de novo* protein synthesis, and once IE proteins have been produced the early and late genes are transcribed and virus replication ensues (Honess and Roizman, 1974; Everett, 1987). The IE proteins ICP4 (Vmw175), ICP0 (Vmw110), and ICP27 (Vmw63) are important regulators of gene expression, although ICP0 is not absolutely required for virus replication (Preston, 1979b; Dixon and Schaffer, 1980; Everett, 1984; O'Hare and Hayward, 1985; Sacks *et al.,* 1985; Stow and Stow, 1986; Sacks and Schaffer, 1987; Sandri-Goldin and Mendoza, 1992). The IE genes are activated by the tegument protein VP16 (Vmw65 or α-TIF), which is incorporated into a multiprotein complex at TAATGARAT (R is a purine nucleotide) sequence elements present in all IE promoters (Post *et al.,* 1981; Campbell *et al.,* 1984; Pellett *et al.,* 1985; McKnight *et al.,* 1987; O'Hare and Goding, 1988; Preston *et al.,* 1988). In addition to VP16 the complex contains the cellular factors Oct-1, which binds to TAATGARAT, and HCF, a large protein which is cleaved to a number of smaller products (Gerster and Roeder, 1988; O'Hare and Goding, 1988; Stern *et al.,* 1989; Katan *et al.,* 1990; Kristie and Sharp, 1990; Wilson *et al.,* 1993). A major consequence of these protein–protein interactions is to bring the C-terminal activating region of VP16 into proximity with components of transcription preinitiation complexes at the IE gene TATA box and cap sites and hence to stimulate IE transcription (Sadowski *et al.,* 1988; Triezenberg *et al.,* 1988).

The importance of activation of IE transcription by VP16 has been demonstrated by analysis of *in*1814, an HSV-1 mutant with a 12-base pair (bp) insertion within the coding sequences for VP16 (Ace *et al.,* 1989). The insertion disrupts a domain of VP16 crucial for interaction

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with Oct-1 and HCF (Ace *et al.,* 1988; Greaves and O'Hare, 1990; Hayes and O'Hare, 1993), so that *in*1814 is impaired for the activation of IE transcription. As a consequence of this impairment, *in*1814 does not initiate productive replication efficiently after infection of tissue culture cells under conditions in which only one virus particle enters a cell; instead, the majority of cells infected with *in*1814 at low m.o.i. retain the viral genome in a quiescent "latent" state, from which gene expression cannot be detected (Harris and Preston, 1991). An apparently similar phenotype is observed after inoculation of *in*1814 into mice, when replication at the site of inoculation and in the ganglion is depressed but neuronal latency is established efficiently (Steiner *et al.,* 1990; Ecob-Prince *et al.,* 1993). In tissue culture cells and in animal models, therefore, *in*1814 replicates poorly and is predisposed to latency.

The protein ICP0 is not absolutely required for virus replication but facilitates the efficient initiation of productive replication when cells are infected at low m.o.i. ICP0 stimulates gene expression from all promoters tested to date in plasmid cotransfection assays, including those controlling HSV-1 IE, early and late HSV-1 genes (Everett, 1984; O'Hare and Hayward, 1985; Cai and Schaffer, 1992; Chen and Silverstein, 1992). These findings suggest a role for ICP0 in activating all classes of gene expression during infection, and in addition the protein may be important in reactivation of HSV from latency by switching on viral genes, including IE genes, in the absence of VP16 (Cai *et al.,* 1993; O'Rourke and O'Hare, 1993). Decreased expression of ICP0 is a major factor determining the phenotype of *in*1814, since HSV-1 mutants lacking functional ICP0 show a restriction similar to that of *in*1814 in tissue culture cells (Stow and Stow, 1986; Sacks *et al.,* 1987; Everett, 1989), and transfection of a plasmid that expresses ICP0 partially complements the replication defect of *in*1814 (Ace *et al.,* 1989). Furthermore, *in*1814 genomes which do not replicate can be sequestered in cells and recovered days later by superinfection of cultures with HSV-1, but only if the superinfecting virus expresses functional ICP0 (Harris and Preston, 1991).

The possibility of using HSV as a vector for gene therapy is currently the focus of many studies, since it may be possible to utilize the ability of HSV to establish latency to achieve retention of the viral genome in neurological tissues, and to use appropriate promoters to direct the long-term expression of foreign gene products and thereby derive therapeutic benefit (Glorioso *et al.,* 1992, 1995; Geller, 1993; Efstathiou and Minson, 1995). A major concern, however, is the known cytotoxicity of HSV. This problem has been investigated by Johnson and coworkers, who have demonstrated that the synthesis of IE proteins, rather than the presence of virus structural proteins, is the main cause of cell killing (Johnson *et al.,* 1992; 1994). Their conclusions are partially based on the analysis of a derivative of *in*1814 which is additionally deleted for ICP4, the major transactivator of virus gene expression. Using this double mutant (named 14ΔH3), significant cell survival was obtained after infection at m.o.i. values that should ensure that virtually all cells in a culture were infected. More recently, Wu *et al.* have shown that tissue culture cells survive infection with mutant d95, which is deleted for the IE genes encoding ICP4, ICP22, and ICP27, for at least 3 days (Wu *et al.,* 1996). Cells infected with d95 did not divide, however, and could not form colonies after subculture and reseeding, suggesting that one or more of the proteins synthesized by d95 was ultimately cytotoxic. It is likely that ICP0 was involved in the inhibition of cellular functions because the protein accumulated in intranuclear bodies in d95-infected cells (Wu *et al.,* 1996).

In the studies reported here, we describe the production and properties of novel host range HSV-1 mutants that were initially constructed to test whether overexpression of ICP0 can compensate for the absence of VP16 function in the context of virus infection, as predicted from studies based on the plasmid transfection approach (Cai and Schaffer, 1992; Chen and Silverstein, 1992). The ICP0 promoter of *in*1814 was replaced with the strong LTR promoter of Moloney murine leukemia virus (Momulv) and the resulting virus, *in*1820, was found to replicate efficiently in BHK cells but to behave in other cell types as if it lacks ICP0. This property was used for the construction and efficient propagation of additional mutants that are conditionally deficient in VP16, ICP0, and ICP4 function and thus fail to produce the three major HSV-1 transactivators. These viruses, the most impaired HSV-1 recombinants in terms of activation of gene expression yet produced, can be used to direct the expression of foreign genes without killing the host cell.

MATERIALS AND METHODS

Plasmids

Plasmid pGX164 is the XhoC fragment from the HSV-1 strain 17 temperature-sensitive mutant *ts*K, which has a mutation in the ICP4 coding sequences (Davison *et al.,* 1984). Plasmids pMC1, encoding VP16, and pMJ101, containing *Escherichia coli* lacZ controlled by the human cytomegalovirus (HCMV) major IE promoter, have been described previously (Campbell *et al.,* 1984; Jamieson *et al.,* 1995). To replace the ICP0 promoter with the Momulv LTR promoter, the starting plasmid was pJR3, which encodes ICP0 (Everett, 1984). Plasmid pJR3 was cleaved with *Nco*I and *Sst*I to remove the 966-bp ICP0 promoter and regulatory region, and a double-stranded oligonucleotide (5′-CATGGGGGTCGTATGCGATATCGGACCTATCGATGGTCCAAGCGCTTGGCACAGATCTTCCGAGCT on one strand, with 4-bp 5′- and 3′-extensions) introduced between the two sites. A 1.5-kbp *Sst*I/*Rsa*I fragment from *Bam*HI k (representing sequences upstream of the ICP0 promoter) was cloned between the adjacent

*Sst*I and *Eco*RI sites of pJR3, to extend the region upstream of the ICP0 coding sequences. The 760-bp *Bam*HI fragment from pM1SP6 (Lang *et al.,* 1983), containing the Momulv LTR promoter, was cloned into the *Bgl*II site introduced by the oligonucleotide (underlined), and a plasmid with the Momulv promoter in the orientation to direct expression of ICP0 was isolated, purified, and named pMo1. This plasmid was modified by removal of the 250 bp of mink DNA upstream of the Momulv LTR, to produce pMo2. Plasmid pMJ88 was constructed by cleavage of pMJ101 with *Hin*dIII and *Xho*I and insertion of a *Hin*dIII/*Sal*I fragment from a modified pMC1NeoPoly(A) (Nicholl and Preston, 1996), to place the neomycin phosphotransferase (npt) coding sequences downstream from the HCMV IE promoter.

Viruses

Mutant *in*1814 and the rescued "revertant" 1814R have been described previously (Ace *et al.,* 1989). *In*1820 was constructed by cotransfecting BHK cells with *in*1814 DNA, together with *Hin*dIII-cleaved pMo1. DNA from plaque isolates was digested with *Xba*I and *Bst*EII, subjected to agarose gel electrophoresis and Southern transfer, and screened by hybridization with a 4.5-kbp *Bst*EII fragment of pMo1 covering the site of insertion. Isolates yielding bands of 3.2 and 1.3 kbp, due to the introduction of an *Xba*I site present in the Momulv promoter, were plaque purified and retested by hybridization. An isolate containing no parental 4.7-kbp fragment was named *in*1820. Mutant *in*1820B was isolated in the same way from an independent transfection, while mutant *in*1820C was prepared in the same way except that pMo2 was used instead of pMo1. The VP16 mutation of *in*1820 was rescued by cotransfection of pMC1 with *in*1820 DNA and purification of viruses containing an intact *Bam*HI f fragment, as determined by cleavage of DNA with *Bam*HI and hybridization to radiolabeled pMC1. A homogeneous isolate was named *in*1825. The Momulv insertion of *in*1825 was rescued by cotransfection of *in*1825 DNA with *Dra*I-cleaved pGX152 (the cloned *Eco*RI b fragment) into BHK cells and subsequent plaque purification on HFL cells. Cleavage with *Xba*I plus *Bst*EII followed by Southern transfer and hybridization using radiolabeled 4.5-kbp *Bst*EII fragment from pMo1 as probe was carried out as described above, this time screening for the presence of a 4.7-kbp fragment. A homogeneous isolate was purified and named 1825R. Mutant *in*1814K was produced by cotransfection of *in*1814 DNA with *Xho*I-cleaved pGX164 and subsequent screening for temperature sensitivity on BHK cells. Two isolates were plaque purified twice and tested for recombination with *ts*K (results not shown). Neither isolate recombined with *ts*K to produce nontemperature sensitive virus, and one was selected for further analysis. DNA from *in*1814K was cotransfected with *Hin*dIII-cleaved pMo1 and a recombinant homozygous for the Momulv LTR was isolated as described above, except that virus was propagated at 31°, to yield *in*1820K. *Sca*I-cleaved pMJ101 was cotransfected with DNA from *in*1814K or *in*1820K and selection for the absence of thymidine kinase (TK), followed by Southern transfer and hybridization, was carried out as described previously (Jamieson *et al.,* 1995). These manipulations produced *in*1894 (from *in*1814K) and *in*1332 (from *in*1820K). Mutant *ts*K/MJ101 was similarly constructed from *ts*K. Mutant *in*1322 was also produced by selection for TK^- viruses, after cotransfection of cells with *in*1820K DNA and *Sca*I-cleaved pMJ88. Rescue of the VP16 mutation of *in*1332 was carried out by cotransfection of *in*1332 DNA with pMC1, as described above, to yield *in*1302. Mutants were routinely tested for responsiveness to hexamethylene bisacetamide (HMBA), to ensure that the VP16 mutation was present (McFarlane *et al.,* 1992) and for plaque formation at 38.5° in the case of ts mutants. Plaques were usually undetectable at 38.5°, indicating that the proportion of revertants was less than 1 in 10^6. Particle concentrations were determined by electron microscopy as described previously (Ace *et al.,* 1989).

Cells

BHK-21 C13 cells were used for propagation of virus stocks, with the addition of HMBA for the first 24–48 hr postinfection where appropriate. HFL cells were Flow 2002 (Flow laboratories). Vero and HeLa cells were obtained from Flow laboratories. Mewo cells, derived from a human melanoma, were provided by Dr A. Davison (MRC Virology Unit, Glasgow), and Rat-1 cells were a gift of A. Ingram (Glasgow University Virology Department). Vero cell lines resistant to G418 were prepared after transfection with pMJ88, as described previously (Nicholl and Preston, 1996).

Cytotoxicity assay

Confluent Vero cell monolayers on 35-mm-diameter plates were infected with 5 PFU of mutant viruses per cell, or mock infected, and incubated at 38.5° for 24 hr. Plates were then trypsinized and cells suspended in 1 ml of culture medium. Ten-fold serial dilutions were made and added to 35-mm-diameter plates, in a total volume of 2 ml. Cells were incubated at 38.5° for 6 days, with a change of growth medium after 3 days, treated with Giemsa stain, and colonies counted.

Histochemical staining for β-galactosidase

Monolayers of Vero or HeLa cells on 35-mm-diameter plates were infected with 5 PFU of *in*1332 per cell, or mock infected, and incubated at 38.5°. At 6 hr postinfection, monolayers were fixed and stained for the presence of β-galactosidase using 5-bromo-4-chloro-3-indolyl β-D-

TABLE 1

Viruses Used in the Study

Name	VP16	ICP0 promoter	ICP4	Insert[a]
*in*1814	−	0[b]	wt	
1814R	+	0	wt	
*in*1820	−	Mo[c]	wt	
*in*1825	+	Mo	wt	
1825R	+	0	wt	
*in*1820B	−	Mo	wt	
*in*1820C	−	Mo	wt	
*in*1814K	−	0	ts	
*in*1894	−	0	ts	HCMV-lacZ
*in*1820K	−	Mo	ts	
*in*1332	−	Mo	ts	HCMV-lacZ
*in*1322	−	Mo	ts	HCMV-npt
*in*1302	+	Mo	ts	HCMV-lacZ
*ts*K/MJ101	+	0	ts	HCMV-lacZ

[a] Insertions at the TK locus.
[b] Normal ICP0 promoter.
[c] Momulv LTR promoter.

galactoside (X-gal) as substrate, as described previously (Jamieson *et al.,* 1995).

Conversion to G418 resistance

Confluent Vero cell monolayers on 35-mm-diameter plates were infected with 1 or 5 PFU of *in*1322 per cell, or mock infected, and incubated at 38.5° with 800 μg of G418 (Geneticin, from Life Technologies, Paisley, Scotland) per milliliter. At 6 hr postinfection, cells were trypsinized and 35-mm-diameter plates reseeded with one-tenth of the cell suspension. Cells were incubated at 38.5° with 500 μg/ml G418, and at various times monolayers were trypsinized and cells counted using a counting chamber. The mean value from four independent determinations was calculated.

Npt assays

Cells were washed with phosphate-buffered saline and lysed with a buffer consisting of 10 m*M* Tris–HCl, 2 m*M* $MgCl_2$, 10 m*M* NaCl, 0.5% (v/v) Nonidet P-40, pH 7.5, and cytoplasmic extracts made as described previously (Preston, 1979a). Npt activity was assayed by incorporation of ^{32}P into neomycin, as described previously (Palmer *et al.,* 1993).

RESULTS

Substitution of the *in*1814 ICP0 promoter

The experiments described here are based on the construction and analysis of HSV-1 mutants, and the salient features of the viruses used are summarized in Table 1. Initially, it was intended to investigate whether substitution of the ICP0 promoter with a strong "enhancer" element compensated for the absence of VP16 function, to yield a virus unable to establish latency in tissue culture or animals. Cotransfection of *in*1814 DNA with pMo1, in which the Momulv LTR promoter replaces the ICP0 promoter, resulted in a high proportion (23/48) of virus plaque isolates containing the substitution, and a second round of plaque purification on BHK cells readily produced a pure homozygous preparation, *in*1820. The ease with which *in*1820 was isolated suggested a selective advantage for the new mutant. The VP16 insertion of *in*1820 was rescued by cotransfection with pMC1 to yield *in*1825, structurally wild-type HSV-1 except for the ICP0 promoter exchange, and the Momulv insertion was then removed by recombination with pGX152 to give 1825R, in which all introduced mutations were rescued.

Viruses were titrated on BHK or HFL cells in the presence or absence of HMBA, which complements the replication defect of *in*1814 by stimulating IE gene expression (McFarlane *et al.,* 1992) (Table 2). To provide a comparison between virus mutants, particle concentrations were determined and the particle:PFU ratios on the two cell lines calculated. Thus, high ratios indicate impairment in the initiation of productive infection. As expected, 1814R preparations contained between 10 and 47 particles per PFU on BHK and HFL cells. Mutant *in*1814 gave values at least 100-fold higher than those of 1814R, with greater impairment on HFL than BHK cells (Ace *et al.,* 1989). In the presence of HMBA, *in*1814 gave a ratio 2.5 (BHK)- or 5.2 (HFL)-fold greater than that of 1814R, in agreement with previous studies which showed that complementation by HMBA underestimates the number of potentially active particles by a factor of approximately 3 (McFarlane *et al.,* 1992; Jamieson *et al.,* 1995). Mutant *in*1820 exhibited a different pattern: on BHK cells it initiated infection 10-fold more efficiently than *in*1814, and with HMBA it formed plaques 3- to 6-fold less efficiently than 1814R. On HFL cells, however, *in*1820 was more restricted than *in*1814, behaving as if deficient in both VP16 and ICP0. Plaque formation on HFL cells was stimulated 100-fold by HMBA but complementation was not complete, as

TABLE 2

Particles:PFU Characteristics of Virus Mutants

		Particle:pfu ratio[a]	
Virus	HMBA	BHK	HFL
1814R	−	19 (11–47)[8]	28 (10–38)[4]
*in*1814	+	48 (15–120)[10]	114 (31–290)[5]
*in*1814	−	4,700 (1,500–10,000)[9]	96,000 (4,400–440,000)[7]
*in*1820	+	120 (40–182)[5]	5,700 (3,000–8,600)[4]
*in*1820	−	314 (150–562)[8]	380,000 (15,000–900,000)[7]
*in*1825	+	11 (10–13)[3]	6,200 (1,200–15,000)[4]
*in*1825	−	31 (17–72)[5]	50,000 (5,800–90,000)[5]
1825R	−	7 (4–11)[4]	13 (8–16)[3]

[a] The range of values is given in parentheses and the number of determinations as a superscript.

TABLE 3

Efficiency of Plaque Formation on Different Cell Types

Virus	HMBA[a]	Relative efficiency of plaque formation					
		BHK	HFL	HeLa	MeWo	Vero	Rat-1
1814R	−	100[b]	63	26	125	197	20
*in*1814	+	100[b]	56	38	100	60	15
*in*1814	−	1.5	0.064	0.43	0.10	1.6	0.006
*in*1820	+	100[b]	1.2	14	6.7	2.1	7.7
*in*1820	−	58	0.016	0.36	0.067	0.15	0.015

[a] HMBA was added at 3 m*M* throughout the titration for BHK cells, at 5 m*M* throughout for HFL, HeLa, MeWo, and Rat-1, and at 3 m*M* between 1 and 6 hr after infection for Vero cells.

[b] The titer on BHK cells (+HMBA for *in*1814 and *in*1820) was defined as 100, and the titers on other cell types were compared to this value for a given virus.

shown by the high particle:PFU ratio of 5,700 (200-fold greater than that of 1814R). Mutant *in*1825 initiated infection in BHK cells essentially as well as 1814R but was restricted in HFL cells, and HMBA increased the efficiency of plaque formation only by 5-fold, as shown previously (McFarlane *et al.,* 1992). The multiple rescuant 1825R initiated infection at least as efficiently as 1814R in both cell types. Substitution of the Momulv LTR for the ICP0 promoter therefore produced a host range mutant, *in*1820, which is less restricted than *in*1814 for replication in BHK cells but more restricted in HFL cells.

To determine which phenotype is observed generally, a range of tissue culture cells was infected with 1814R, *in*1814, or *in*1820 (Table 3). Although variation was found in the relative plating efficiencies of *in*1814 and *in*1820 between cell types, as noted previously (Ace *et al.,* 1989), the titer of *in*1820 without HMBA did not approach the fully complemented value (BHK cells with HMBA) in any of the cultures examined except BHK. Mutant *in*1820 was therefore restricted for initiation of replication in all of the additional cell types tested including the rodent line rat-1. The presence of HMBA only partially complemented plaque formation by *in*1820, and again the degree of enhancement varied between cell types.

The observation that 1825R initiates infection of HFL cells as efficiently as 1814R suggests that the promoter exchange, rather than other fortuitous mutations, is responsible for the unusual phenotype of *in*1820. To confirm that this is the case, a second isolate, *in*1820B, was made from *in*1814 in an independent cotransfection. In addition, because the Momulv insert used to create *in*1820 also contains 250 bp of mink genomic DNA upstream of the LTR (Lang *et al.,* 1983), a virus lacking the mink portion, *in*1820C, was constructed. The new mutants exhibited the same host range as *in*1820 (Table 4), demonstrating that the Momulv LTR is responsible for the novel phenotype of *in*1820.

In addition, the unlikely possibility that a mutation had arisen during the construction of pMo1, such that transactivation by ICP0 was improved in BHK but impaired in HFL cells, was considered. The ICP0 coding sequences were cloned from *in*1825 DNA as a 5345-bp *Nco*I/*Pst*I fragment and used to construct pMJ94, which is identical to pJR3 (apart from the absence of a 200-bp *Pst*I fragment downstream of the ICP0 sequences). In cotransfection assays, pJR3 and pMJ94 stimulated β-galactosidase expression from pCMVβ-gal (lacZ controlled by the HCMV major IE promoter) to the same extent (approximately fourfold) in both BHK and HFL cells (results not shown). Therefore, the host range phenotypes of *in*1820 and *in*1825 are not due to a mutation in the ICP0 coding sequences.

Following the *rationale* for the construction of *in*1820, it is likely that alterations to the expression of ICP0 account for the phenotype of the mutant. This conclusion is supported by two observations: first, reversion of the mutations of *in*1820 results in a virus (1825R) that is indistinguishable from 1814R and, second, the particle:PFU ratio of *in*1825 in restricted cells is within the range of that reported for the ICP0 null mutant *dl*1403 (Stow and Stow, 1986; Everett, 1989). Recent studies, however, have shown the existence of transcripts

TABLE 4

Comparison of *in*1820, *in*1820B, and *in*1820C

Virus	HMBA	Relative efficiency of plaque formation	
		BHK	HFL
*in*1820	+	100[a]	3.8
*in*1820	−	44	0.060
*in*1820B	+	100[a]	3.0
*in*1820B	−	41	0.024
*in*1820C	+	100[a]	3.0
*in*1820C	−	32	0.024

[a] The titer on BHK cells, with HMBA, was defined as 100 and other titers for a given virus were compared to this value.

TABLE 5

Mutants *in*1825 and *dl*1403 Do Not Cross Complement[a]

Virus 1	Particles ($\times 10^5$)	Virus 2	Particles ($\times 10^5$)	Plaques[b]
*dl*1403	1.25			14 (9–16)
*dl*1403	2.5			40 (34–48)
*dl*1403	5.0			92 (86–102)
*in*1825	1.25			36 (33–40)
*in*1825	2.5			73 (64–84)
*in*1825	5.0			120 (107–138)
*ts*K	2.5			0
*ts*1201	2.5			0
*dl*1403	1.25	*in*1825	1.25	59 (46–74)
*dl*1403	2.5	*in*1825	2.5	113 (104–116)
*dl*1403	2.5	*ts*K[c]	0.25	76 (65–83)
*dl*1403	2.5	*ts*K	2.5	>300
*dl*1403	2.5	*ts*1201	0.25	106 (99–109)
*dl*1403	2.5	*ts*1201	2.5	>300
*in*1825	2.5	*ts*K	0.25	120 (107–138)
*in*1825	2.5	*ts*K	2.5	>300
*in*1825	2.5	*ts*1201	0.25	145 (143–146)
*in*1825	2.5	*ts*1201	2.5	>300
*ts*K	0.25	*ts*1201	2.5	124 (112–135)
*ts*K	2.5	*ts*1201	0.25	140 (139–140)
*ts*K	2.5	*ts*1201	2.5	>300

[a] Monolayers of 8×10^5 HFL cells were coninfected and plaques formed at 38.5° were scored.

[b] The range of values from 3 to 6 determinations is given in parentheses.

[c] The *ts*K and *ts*1201 preparations had particle:PFU ratios of 8.8 and 7.9, respectively (31° titers). This information can be used to estimate that the *dl*1403 and *in*1825 preparations contained 19 and 16 particles per complementing unit, respectively (for example, 0.25×10^5 particles of *ts*K represents 2.8×10^3 PFU or 1 cell in 285 infected with a PFU; coinfection with 2.5×10^5 particles of *dl*1403 gave an increase of 36 in the *dl*1403 titer, thus 2.5×10^5 *dl*1403 particles represents 1×10^4 [36×285] complementing units. The analogous calculation for *dl*1403 and *ts*1201 gives 1.6×10^4 complementing units from 2.5×10^5 PFU *dl*1403 particles). Using these values, it can be calculated that cultures which received 2×10^5 PFU each of *dl*1403 and *in*1825 contained 230 cells dually infected with a complementing unit of each virus. In the control experiment, coinfection with 2.5×10^5 particles of *ts*K and 0.25×10^5 particles of *ts*1201 was calculated to result in 56 cells receiving a PFU of each virus, thus the observed value of 124 plaques demonstrates that complementation was as efficient as predicted.

(named L/STs) that are transcribed divergently from the mRNA encoding ICP0, and the presumed promoter for these transcripts was also removed in the construction of *in*1820 (Yeh and Schaffer, 1993; Bohensky *et al.,* 1995). To confirm that the reduced efficiency of plaque formation in HFL cells is due to the effective absence of ICP0, complementation between *in*1825 and the ICP0 deletion mutant *dl*1403 was investigated (Table 5). This experiment is less sensitive than a classical complementation analysis because the deletion of ICP0 is not a "tight" mutation and thus there is a background of replication from those viruses which enter the lytic cycle, even at low m.o.i. Monolayers of HFL cells were coinfected with various pairings of mutants, including *ts*K and the ICP24 mutant *ts*1201 (Preston *et al.,* 1983), and plaque formation at 38.5° determined. Mixed infections containing 1.25×10^5 particles each of *dl*1403 and *in*1825 gave a number of plaques (59) approximately equal to the mean of that exhibited by 2.5×10^5 particles of the viruses individually (40 and 73) and, similarly, mixing 2.5×10^5 particles of each mutant gave a value (113) essentially equivalent to the mean of 5×10^5 particles of *dl*1403 and *in*1825 alone (92 and 120). Control experiments showed that complementation of *dl*1403 could be detected even with only 2.5×10^4 particles of *ts*K or *ts*1201, both of which produce ICP0. This experiment demonstrates that *in*1825 did not produce amounts of ICP0 sufficient to complement *dl*1403, and hence that the lack of functionally adequate levels of ICP0 is the cause of the restricted initiation of replication in HFL cells, and presumably other cell types apart from BHK.

Construction of HSV-1 mutants with multiple defects in IE gene function

In nearly all cell types tested *in*1820 is effectively devoid of VP16 and ICP0 activity, yet it can readily be propagated in BHK cells in the presence of HMBA since under these conditions it replicates almost as efficiently as wild-type HSV-1. When cultures are infected under conditions in which individual cells receive 1 particle of *in*1814 or *in*1820, most cells survive infection and harbor the virus in a quiescent state which resembles latency in some respects (Harris and Preston, 1991; Jamieson *et al.,* 1995). At higher m.o.i., however, cell destruction occurs, limiting the value of these mutants as potential vectors. Since expression of IE proteins is mainly responsible for cell killing (Johnson *et al.,* 1994), we constructed a virus which is conditionally deficient in VP16, ICP0, and ICP4 functions. Mutant *in*1814K was first produced, and this was used as a template for the formation of *in*1820K. In BHK cells at 31° in the presence of HMBA, *in*1820K can be propagated essentially as wild-type HSV-1 since none of the three mutations are operative, but at 38.5° in HFL (or other cell types) without HMBA the virus is effectively negative for VP16, ICP0, and ICP4 functions. This is the most restricted HSV mutant in terms of transcription activators available to date, and in the following sections the properties of this novel mutant, with particular emphasis on its possible value as a prototype vector, are described.

Cytotoxicity

It was possible to infect a number of cell types with 5 PFU of *in*1820K per cell without visible cpe after 24 hr at 38.5°, whereas *in*1814K under the same conditions caused alterations to cell morphology, particularly rounding of cells (results not shown). To apply a more stringent evaluation of cytotoxicity, Vero cultures were infected with 5 PFU of various viruses per cell, incubated at 38.5° for 24 hr, trypsinized, serially diluted and replated

TABLE 6

Cytotoxicity of Virus Mutants[a]

Virus	Plating efficiency (%)[b]
*in*1332	91 (77–100)
*in*1894	13 (8–17)
*in*1302	3 (1–5)
*ts*K/MJ101	0.4 (0.1–0.6)

[a] Vero cell monolayers were infected with 5 PFU of various viruses per cell and incubated at 38.5° for 24 hr. Monolayers were trypsinized, serially diluted, and plated out. Colonies formed after 6 days were counted and expressed as a percentage of the number derived from mock-infected cells. The efficiency of plating of mock-infected cells was approximately 100%.

[b] The mean values from three independent experiments are presented, with the ranges given in parentheses.

at 38.5°. Colonies were counted after 6 days (Table 6). Mutant *in*1332 only marginally reduced plating efficiency at m.o.i. of 5 PFU per cell, demonstrating that the majority of cells infected in this way survived infection. Cell viability of approximately 100% was also observed when subculture was carried out at 6 hr after infection of Vero cells, and when HeLa or human 143TK$^-$ cells were infected with 5 PFU of *in*1332 for 24 hr (results not shown). Infection with mutant *in*1894 reduced colony formation by a factor of approximately 7, demonstrating that ICP0 function contributed to cytotoxicity. Mutant *ts*K/MJ101 killed all cells estimated to receive at least 1 PFU of virus. The mutant *in*1302, in which the VP16 mutation was rescued from *in*1332, regained significant cytotoxicity, demonstrating that the absence of VP16 function and consequent reduction in IE gene expression was a crucial factor in the reduced cytotoxicity of *in*1820K based mutants. This observation formally rules out the possibility that other fortuitous mutations in *in*1814 or its derivatives are responsible for the reduced cell killing by *in*1820K (or 14ΔH3, described by Johnson *et al.,* 1994).

Gene expression

To determine the extent to which vectors based on *in*1820K express foreign genes, reporter sequences were cloned under the control of the HCMV major IE promoter and subsequently recombined into the viral genome. Vero and HeLa monolayers were infected with 5 PFU per cell of *in*1332, which contains the lacZ gene controlled by the HCMV IE promoter, and incubated at 38.5° for 6 hr. Upon histochemical staining, β-galactosidase was present in almost all cells in the culture, generally at low but detectable levels although expression was heterogeneous in that some cells expressed the enzyme more efficiently than the majority (Fig. 1). Therefore, it is possible to establish conditions in which effectively all cells remain viable and express β-galactosidase.

To investigate whether *in*1820K-based mutants could be used to produce significant functional levels of foreign proteins in infected cells, the npt gene controlled by the HCMV IE promoter was cloned into the TK locus of *in*1820K, to yield *in*1322. Vero cell monolayers were infected with *in*1322 in the presence of G418, incubated at 38.5° for 6 hr and then trypsinized and plated at 38.5° with G418 present, at a split ratio of 1:10. Infected cell cultures replicated rapidly, whereas mock-infected cultures did not (Fig. 2A). At 2 days after subculture, monolayers infected with 5 PFU per cell were largely normal in appearance (Fig. 2D), while mock-infected cells displayed characteristic alterations due to killing by G418 (Fig. 2B). Cells derived from infected cultures remained predominantly morphologically normal for a further 2 days, although increasing numbers cells showing G418-induced cytopathology became apparent. Similar results were obtained when the initial m.o.i. was 1 PFU of *in*1322 per cell (Fig. 2C), although greater cell degeneration was observed at 3 days after subculture. Therefore, amounts of npt sufficient to protect cells from G418 toxicity for at least 2 days were produced after infection of Vero cultures with 5 PFU of *in*1322 per cell.

The degree and duration of protection from the toxic effects of G418 after infection with *in*1322 were somewhat unexpected, for two reasons. First, when quiescent in tissue culture cells *in*1814-based mutants do not replicate their genomes as the host cell divides (Russell and Preston, 1986; Jamieson *et al.,* 1995), thus many of the cells in cultures that had reached confluence may not have contained a viral genome. Second, expression of lacZ-specific RNA from the HCMV IE promoter is transient in cells infected with *in*1332 (C. M. Preston, unpublished results), thus prolonged expression of npt from the *in*1322 genome would not have been anticipated. The production of npt was therefore assayed in cultures infected with *in*1322, and as a control the enzyme levels in Vero cells stably transformed to G418 resistance by pMJ88 was also determined (Fig. 3). The major period of npt synthesis occurred during the initial stages of infection but enzyme levels remained above those in the transformed cell line until 3 days postinfection. Therefore, infection with *in*1322 results in the retention of functionally significant levels of npt for up to 3 days postinfection, explaining the successful short term alteration of cell phenotype shown in Fig. 2.

DISCUSSION

In making an apparently straightforward change to the *in*1814 genome, we have produced a virus mutant with an unexpectedly complex host range phenotype: in BHK cells, despite the lack of VP16 function, *in*1820 initiated replication more efficiently than *in*1814, whereas in all other cell types tested *in*1820 was more impaired than *in*1814. Our results demonstrate that the restricted phenotypes of *in*1820 (compared with *in*1814) and *in*1825

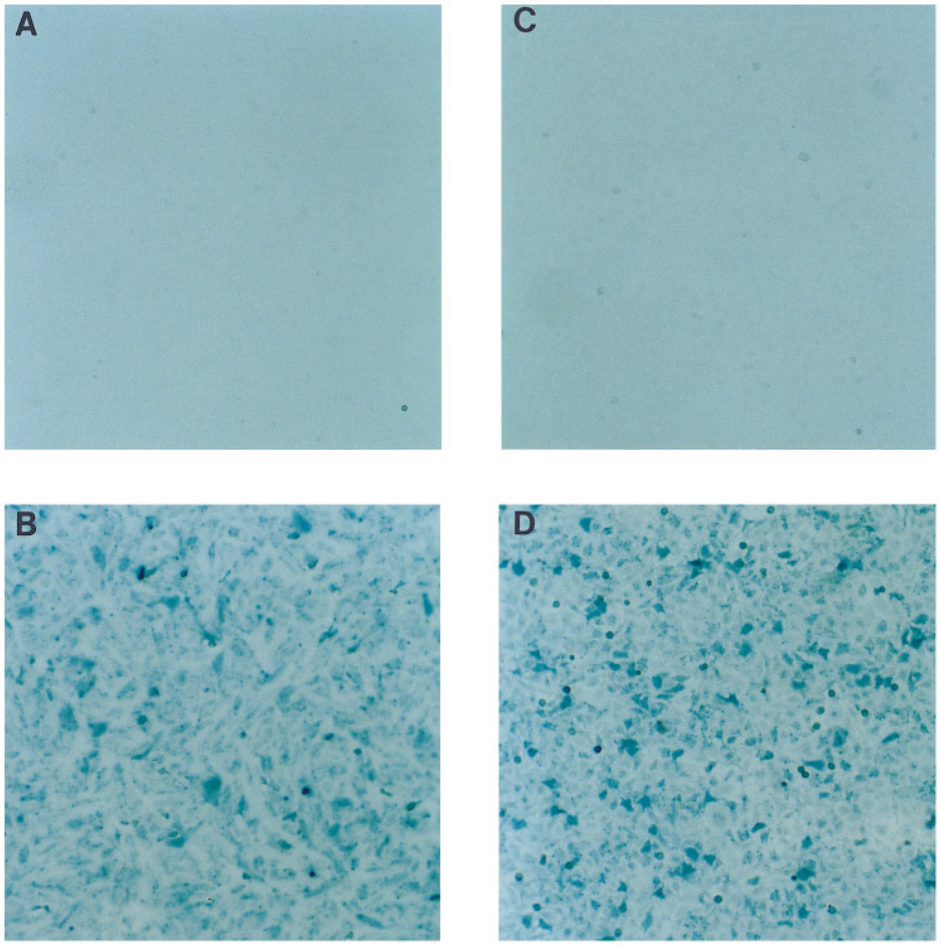

FIG. 1. Production of β-galactosidase. Vero (A and B) or HeLa (C and D) monolayers were mock infected (A and C) or infected with 5 PFU of *in*1332 per cell (B and D) and stained for the presence of β-galactosidase after incubation at 38.5° for 6 hr. No positive cells were detectable on mock-infected cultures, whereas nearly all cells expressed β-galactosidase at low levels in infected cultures. Magnification, $\times$200.

(compared with 1814R) are due to the absence of functional levels of ICP0 and thus failure to activate gene expression efficiently. This conclusion is based on three lines of evidence: first, rescue of the ICP0 promoter exchange overcomes the restriction of *in*1825; second, *in*1825 has the same degree of impairment as *dl*1403, based on their similar particle:PFU characteristics; and, third, *dl*1403 and *in*1825 do not cross complement. It is clear that the surprising phenotype of *in*1820 is due to the Momulv insertion since mutants with the same properties have essentially been constructed independently on three additional occasions (in the production of *in*1820B, *in*1820C, and *in*1820K). Furthermore, the possibility of a host range change in the ICP0 protein was excluded by the observation that the gene cloned from *in*1825 activated gene expression normally. The Momulv LTR is not regulated as a viral IE promoter during infection of HFL cells at high m.o.i. (Jamieson *et al.,* 1995), thus it is likely that insufficient amounts of IE proteins, especially ICP0 itself, are produced to activate the Momulv promoter and consequently the *in*1820 genome is converted to its quiescent form, in which expression of viral genes does not occur (Jamieson *et al.,* 1995). The results demonstrate that the effect of alteration of the regulation of an HSV-1 gene can be as dramatic as that of deleting the gene altogether, thus it may be possible to use this concept to devise new ways of obtaining host or cell-specific expression of foreign genes cloned into HSV-1 vectors.

In an experiment to determine the effect of the ICP0 promoter substitution on virulence after inoculation into mice via the footpad, *in*1825 was found to produce approximately 10-fold fewer neurons positive for HSV-1 antigens than 1825R at 5 days after inoculation, and a similarly reduced number of neurons positive for the major latency-associated transcript during latency (C. M. Preston and M. S. Ecob-Prince, unpublished results). The restricted phenotype exhibited in most tissue culture cells was therefore also observed in mice (even though the Momulv LTR is considered to be adapted for expression in rodent cells).

From the nature of the insertion used to construct

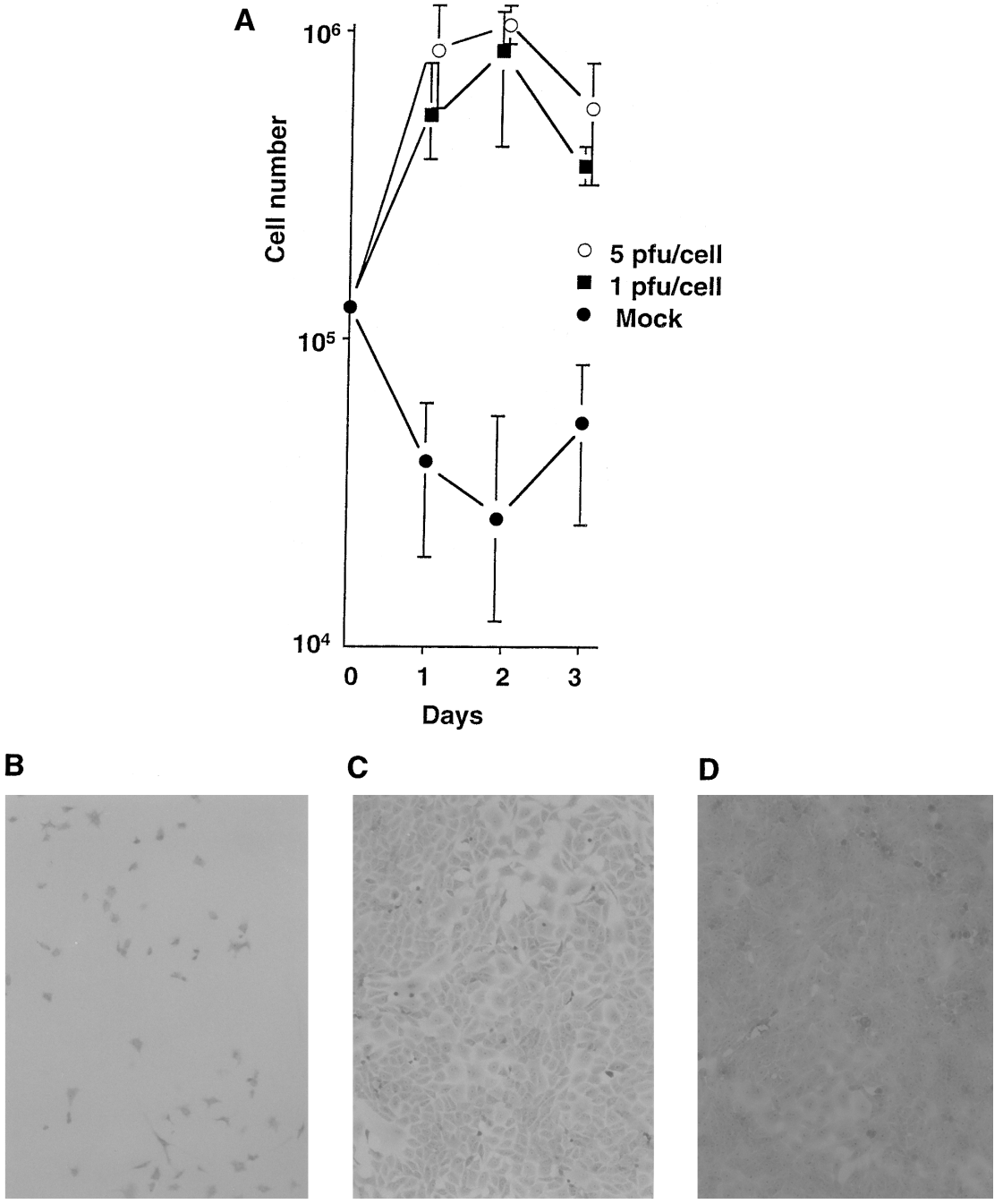

FIG. 2. Production of npt. Vero cells were mock infected or infected with 1 or 5 PFU of *in*1322 per cell. After incubation at 38.5° for 6 hr in the presence of G418, monolayers were trypsinized and cells replated at one-tenth the original density, with G418 present. Cells were counted at various times after subculture (A), and photographs were taken of mock-infected cultures (B) or cultures infected with 1 (C) or 5 (D) PFU of *in*1322 per cell. Magnification, ×200.

*in*1820 and the logic of the experimental approach, it is likely that the improved replication in BHK cells of this mutant, compared with that of *in*1814, is due to greater synthesis of ICP0 and hence compensation for the lack of IE gene activation by VP16. This hypothesis predicts that the Momulv promoter is more active than the ICP0 promoter in BHK cells but that the converse is true in other cell types. To demonstrate that this is the case, it would be necessary to compare ICP0-specific RNA levels under conditions in which the host range phenotype is observed, that is, at very early times after infection with *in*1814 and *in*1820 at low m.o.i. This is difficult, however, for two reasons. First, once ICP4 has been produced, powerful activation of gene expression ensues (due to ICP4 and the consequent increase in the level of ICP0) and thus the relative amounts of ICP0-specific mRNA in

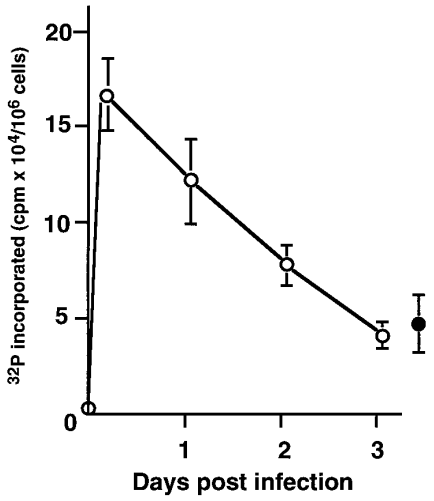

FIG. 3. Production of npt. Vero cell monolayers were infected with 5 PFU of *in*1322 per cell and maintained at 38.5°. At various times duplicate monlayers were harvested and assayed for npt activity (○). In addition, extracts from monolayers of Vero cells transformed to G418 resistance by pMJ88 were assayed (●).

BHK and HFL cells would simply reflect the number of cells destined to enter lytic replication rather than the initial utilization of the Momulv and ICP0 promoters by cellular transcription factors. Second, attempts to measure gene expression under IE conditions are confounded by the observation that inhibition of protein synthesis itself influences promoter activity (C. M. Preston, unpublished observations). Other explanations are possible for the host range phenotype of *in*1820. There may be a factor in BHK cells which mimics the activity of ICP0, as exists in U2OS cells (Yao and Schaffer, 1995), although if this is the case the putative factor must act preferentially on viruses with the Momulv promoter insertion because *in*1825 forms plaques on BHK cells much more efficiently than *dl*1403 (Stow and Stow, 1986). It is possible that alterations to the synthesis of L/STs transcripts and resultant effects on the synthesis of the predicted product, the ORF P protein, influence virus replication in BHK cells in an undetermined way (Yeh and Schaffer, 1993; Bohensky *et al.,* 1995; Lagunoff and Roizman, 1995). It is also noteworthy that the Momulv LTR contains sequences that confer long term expression to the LAT promoter during latency in mice (Lokensgard *et al.,* 1994): perhaps the same property is important for prolonging production of ICP0 in BHK cells.

The preferential replication of *in*1820 in BHK cells is very useful experimentally since it enables mutants that are devoid of both VP16 and ICP0 activity to be propagated almost as efficiently as wild-type HSV-1. This has permitted the construction of novel mutants that are defective in the three major HSV-1-specified transactivators and an assessment of their potential as prototype vectors. The *in*1820K-based mutants were significantly less cytotoxic than *in*1814K counterparts, demonstrating that even the small amounts of ICP0 produced in the absence of VP16 function contribute to cytotoxicity. In tests in which HFL cells were infected and the increase in cell numbers after 3 days was measured, infection with *in*1814K-derived viruses gave results similar to those of Johnson *et al.* (1994) using the same approach, whereas *in*1820K was much less cytotoxic (R. Mabbs and C. M. Preston, unpublished results). We consider, however, that determination of plating efficiency is a more stringent criterion for the estimation of cell viability, and that the ability of at least 90% of cells infected with 5 PFU of *in*1332 to reseed demonstrates that construction of the new mutants has significantly overcome the problem of cytotoxicity, at least in tissue culture cells. The recently described mutant d95 (Wu *et al.,* 1996) is in many ways complementary to *in*1820K, since VP16 and ICP0 functions are retained but ICP4, ICP22, and ICP27 are removed. Consequently, cells infected with d95 express ICP0 and other viral proteins for 3 days, but cease DNA synthesis and are killed, presumably due to the accumulation of ICP0. Our results confirm that ICP0 is cytotoxic even at the low levels made in the absence of VP16 function (i.e., by the *in*1814K derivative *in*1894), and emphasize that reduction of IE protein synthesis is important for cell survival.

The demonstration that *in*1322 can convert an entire cell population to G418 resistance constitutes "proof of principle" that HSV vectors can transfer foreign proteins to tissue culture cells in quantities sufficient to confer a phenotypic alteration without significant toxicity from the vector itself. This property of the *in*1820K mutants may be useful in basic studies designed to analyze relatively short term (up to 48 hr) effects of gene products. The ability of HSV to infect a wide variety of cell types, coupled with the absence of cytotoxicity, provides advantages over many other virus vectors, and the possibility of infecting all cells in a culture gives the potential for more efficient gene transfer than can be achieved by transfection and other chemical methods. In addition, *in* 1820K may be useful as a noncytotoxic helper for the production of HSV-based amplicons. For application in human gene therapy, it will be necessary to couple long-term expression of therapeutic genes with the advantages presented by mutants multiply impaired for expression of IE products.

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